ARTICLE

Improving Performance of a SARS-CoV-2 RT-LAMP Assay for Use With a Portable Isothermal Fluorimeter: Towards a Point-of-Care Molecular Testing Strategy

Mary E. Natoli,[1]# Kathryn A. Kundrod,[1]# Megan M. Chang,[1] Chelsey A. Smith,[1] Sai Paul,[1] Jackson B. Coole,[1] Nathaniel G. Butlin,[2] Nathan A. Tanner,[3] Ellen Baker,[4] Kathleen M. Schmeler,[4] and Rebecca Richards-Kortum[1]

[1]Department of Bioengineering, Rice University, Houston, Texas, USA; [2]Axxin Pty Ltd, Fairfield, Australia; [3]New England Biolabs, Ipswich, Massachusetts, USA; and [4]Department of Gynecologic Oncology and Reproductive Medicine, MD Anderson Cancer Center, Houston, Texas, USA

Frequent and accessible testing is a critical tool to contain the spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). To develop low-cost rapid tests, many researchers have used reverse transcription loop-mediated isothermal amplification (RT-LAMP) with fluorescent readout. Fluorescent LAMP-based assays can be performed using cost-effective, portable, isothermal instruments that are simpler to use and more rugged than polymerase chain reaction (PCR) instruments. However, false-positive results due to nonspecific priming and amplification have been reported for a number of LAMP-based assays. In this report, we implemented a RT-LAMP assay for SARS-CoV-2 on a portable isothermal fluorimeter and a traditional thermocycler; nonspecific amplification was not observed using the thermocycler but did occur frequently with the isothermal fluorimeter. We explored 4 strategies to optimize the SARS-CoV-2 RT-LAMP assay for use with an isothermal fluorimeter and found that overlaying the reaction with mineral oil and including the enzyme *Tte* UvrD helicase in the reaction eliminated the problem. We anticipate these results and strategies will be relevant for use with a wide range of portable isothermal instruments.

INTRODUCTION

Loop-mediated isothermal amplification (LAMP) is a promising technology to detect severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in clinical samples, with 9 LAMP-based tests having received emergency use authorization as of April 2021 and at least 2 scientific advisory groups recommending its use.[1,2] However, LAMP reactions are known to produce nonspecific amplification products due to the use of 6 oligonucleotide primers at high concentrations and the continuous amplification that takes place during isothermal incubation.[3] LAMP reaction mixes are available in "Warm-Start" formulations (NEB E1700) that include an intercalating fluorescent dye and may be assembled easily at room temperature. However, intercalating dyes produce a signal that is proportional to the total amount of double-stranded DNA, and nonspecific products cannot be easily distinguished from true amplification of the desired target. It is advantageous to have strategies to eliminate false-positive results and encourage only on-target amplification when using reverse transcription (RT)-LAMP assays based on intercalating fluorescent dyes.

In this article, we describe performance of an RT-LAMP assay for SARS-CoV-2 that was optimized for a commercial thermocycler and then implemented on a portable isothermal fluorimeter without assay modifications. We then describe 4 strategies to reduce false-positive results observed on the portable device: overlaying a reaction with mineral oil, incorporating helicase, mixing during amplification, and performing a brief vortex and centrifuge prior to the reaction.

The use of mineral oil or other substrates to overlay nucleic acid amplifications had been well established before lid heating was a common feature of thermocyclers, because it prevents evaporation that would lead to changes in both the reaction temperature and the effective concentration of primers and enzymes in the reaction. Mineral oil has been frequently used to overlay reactions in recent reports of LAMP-based assays in cases in which thermocyclers or even reaction tubes were not being used.[4,5] Therefore, we chose to use PCR-grade mineral oil to overlay RT-LAMP reactions when they were performed in the Axxin T8-ISO (Axxin Pty Ltd, Fairfield, Australia). The enzyme *Tte* UvrD helicase was developed by New England Biolabs (NEB; Ipswich, MA) specifically to reduce nontemplate amplification in LAMP assays.[6–9] Helicase functions to unwind double-stranded DNA, reducing both spurious and specific products. Specific products are typically much more abundant than spurious products, so the inclusion of this enzyme can effectively suppress the detection of spurious products.[10]

Finally, agitation is known to increase the speed and lower the limit of detection of other isothermal amplification reactions.[11,12] We hypothesized that either continuous agitation or 1 prereaction vortex and centrifuge of our RT-LAMP reactions could improve temperature homogeneity and reduce the likelihood of nonspecific primer interactions, particularly
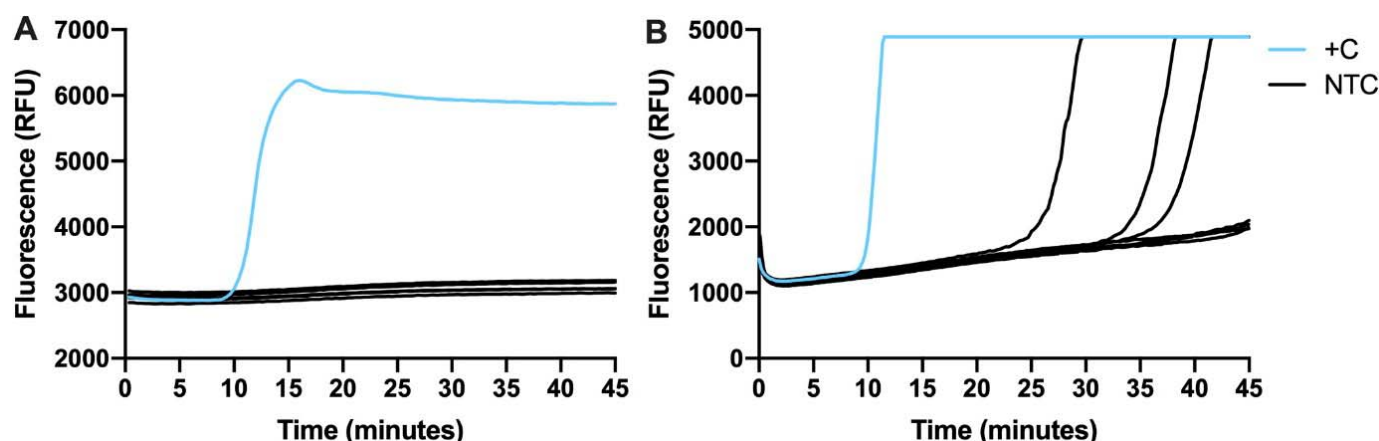
FIGURE 1

Fluorescence *vs.* time for 7 no-template control (NTC) and 1 positive control (+C) RT-LAMP reaction prepared from the same master mix and run on (A) the BioRad CFX96 in 25-μL reaction volumes and (B) the Axxin T8-ISO in 50-μL reaction volumes. Reactions contain either water (NTC) or 10 copies/μL of synthetic SARS-CoV-2 RNA.

due to the larger reaction volume of 50 μL required by the Axxin T8.[13] Axxin molecular diagnostic instruments allow continuous mixing by including a magnet on the optical module, which raises and drops a magnetic ball included in the reaction each time the fluorescence reader passes a tube. Therefore, continuous agitation was accomplished in this study by including a magnetic steel ball bearing in each reaction, and a single prereaction vortex and centrifuge was investigated as a fourth and final strategy to reduce nontemplate amplification.

MATERIALS AND METHODS/EXPERIMENTAL

Instruments and Protocol

Point-of-care instruments for SARS-CoV-2 diagnostic testing should be affordable, portable, and easy to use without specialized training. For real-time detection of fluorescent RT-LAMP reactions, a point-of-care instrument should heat the sample to a predefined temperature, accurately monitor fluorescence *vs.* time, and automatically interpret results as positive or negative. We selected a commercially available instrument—the Axxin T8-ISO—that met these criteria off-the-shelf and without customization for this study.

The latest T8-ISO software (version 3.3.00-383) was installed and test protocols were developed with the provided PC desktop software (Axxin, version 2.8.0.1). Reactions were run at 65°C. The FAM channel was set to 7% pulse-width modulation and readings were taken every 10 seconds; the second HEX channel was not used. Reactions were considered positive if the relative fluorescence intensity reached 2500 mV within 20 minutes. Results were compared with those obtained with a Bio-Rad CFX96 Touch thermocycler (Bio-Rad, Hercules, CA) and its associated CFX Maestro software (version 4.1.2433.1219). All reactions were run at 65°C for 45 minutes in a Bio-Rad CFX96 Touch with or without lid heating set at 105°C. The positive threshold for the Bio-Rad was considered to be a fluorescence increase of 500 relative

fluorescence units from the baseline fluorescence value within 45 minutes.

The oligonucleotide primers (oligos) for the RT-LAMP assay were designed by NEB[14] to target the N2 and E1 genes and by Rabe and Cepko[15] to target the Orf1 gene of the SARS-CoV-2 genome. All oligos were ordered from Integrated DNA Technologies, Inc (Coralville, IA) and resuspended in 1X Tris-EDTA (TE) buffer at a 1-mM concentration. Oligos were combined to make 1000 μL of a 25X mix as follows: 40 μL FIP, 40 μL BIP, 5 μL F3, 5 μL B3, 10 μL LF, 10 μL LB, and 890 μL nuclease-free water.

All RT-LAMP reagents were purchased from NEB (E1700). Standard reaction conditions for the thermocycler include 5 μL of sample in a total reaction volume of 25 μL. Each 25-μL reaction contained 12.5 μL of 2X master mix, 1 μL of 25X N2 primer mix,[14] 1 μL of 25X E1 primer mix,[14] 1 μL of 25X As1e primer mix,[15] and 0.5 μL of 50X fluorescent dye (NEB B1700). Standard reactions were supplemented to 20 μL with nuclease-free water, and then 5 μL of RNA template were added in low-profile 8-tube strips (Bio-Rad, TLS0801) with flat, ultra-clear caps (Bio-Rad TCS0803). All reactions run on the BioRad CFX96 were performed with these specifications, except for the direct comparison between the 2 instruments (**Fig. 1**), in which the dye concentration typically used for the Axxin T8-ISO (0.5 μL of 5X dye per reaction), described in the following paragraph, was used for BioRad reactions as well.

In reactions run on the Axxin T8-ISO, 0.5 μL of 5X fluorescent dye (NEB B1700) was used.[16] In addition, due to the instrument specification for reaction volume of a minimum of 30 μL, reaction volumes of 50 μL were used on the Axxin T8-ISO. In these reactions, the volume of each component of the RT-LAMP assay described in the previous paragraph was doubled, and reactions were assembled in high-profile 8-tube strips (Axygen PCR-0208-C) with domed caps (Bio-Rad TCS0801). Also, 0.2 μL of *Tte* UvrD Helicase (NEB,

M1202S) was included in some reactions on the Axxin T8-ISO.

Molecular biology-grade mineral oil was purchased from Sigma-Aldrich (69794); 2-mm grade 100 AISI 420 stainless steel ball bearings were obtained from SimplyBearings (Leigh, UK). Samples that were vortexed were agitated at maximum speed for approximately 3–5 seconds, then quickly spun in a microcentrifuge. Samples mixed by pipetting were pipetted up and down 3–5 times.

Viral RNA sequences used in this study were purified RNA controls from Twist Biosciences (SKU 102019). Upon receipt, Twist Biosciences control RNA was quantified via RT-qPCR against SARS-CoV-2 genomic RNA (ATCC VR-1991D), then stored in single-use aliquots of 5 μL each at −80° C until use. Upon use, each aliquot was diluted in nuclease-free water, kept on ice, and used within 4 hours.

Clean Reaction Setup

All amplification reactions were assembled and sealed prior to amplification in a dedicated preamplification room that was regularly decontaminated with 10% bleach prepared daily and had limited personnel access. Once reactions were run, reaction tubes were discarded without opening to prevent postamplification contamination of future reactions.

Clinical Sample Collection and Lysis

All lysis buffer solutions were made from molecular grade reagents. TCEP/EDTA/NaOH/GuHCl buffer was used at a concentration of 5X. First, a concentrated TCEP/EDTA/NaOH buffer was adapted from Rabe and Cepko[15] as follows: 358 mg of TCEP-HCl (Millipore Sigma 580567) was dissolved in 568 nuclease-free water. Next, 1 mL of 0.5 M EDTA pH 8 (ThermoFisher Scientific AM9260G) and 575 μL 10N NaOH (Fisher Scientific SS267) were added. Also, 2009 mg of GuHCl (Promega H5381 or H5383, used interchangeably) was dissolved into 1.5 mL nuclease-free water, yielding 3 mL of 7-M GuHCl; 2857 μL of 7-M GuHCl were added to the solution containing TCEP-HCl, EDTA, and NaOH, bringing the total concentrated lysis buffer volume to 5.0 mL.

When used with nasopharyngeal swabs, the concentrated lysis buffer was diluted 1:20 in nuclease-free water, yielding the "swab lysis buffer"; 300 μL of swab lysis buffer was aliquoted into each 1.5-mL collection tube (Sarstedt 72.692.405). When used with saliva, 5 mL of concentrated lysis buffer was added to 10 mL of Tris pH 8.0 (Invitrogen AM9855G), yielding the "saliva lysis buffer." Saliva collection tubes were not preloaded with lysis buffer. All solutions were sterile-filtered through a 0.2-μm filter (Pall Life Sciences Acrodisc 4652), then stored at 4°C for up to 7 days.

Nasal and nasopharyngeal swabs were collected under a protocol approved by the institutional review boards at the University of Texas MD Anderson Cancer Center and Rice University. A medical provider collected nasopharyngeal swabs (Fisher Scientific 23-349-822 or MedicoSwab FS-N96000) into 300 μL of diluted swab lysis buffer. The swab was cut with clean scissors at the top of the tube, leaving the swab tip in buffer. Participants self-collected saliva by passively drooling into a sterile 5-mL tube (MTC Bio C2540). Samples were transported to Rice University on ice and were tested on the Axxin T8-ISO and Bio-Rad CFX96 Touch on the day of collection.

To test samples upon receipt, tubes containing swabs and swab lysis buffer were surface decontaminated, vortexed for 20 seconds, briefly spun in a microcentrifuge, and heated at 95°C for 5 minutes in a dry heat block (VWR 10153-348) for lysis. To lyse saliva samples, 255 μL of saliva were combined with 45 μL of saliva lysis buffer; the mixture was vortexed and heated at 95°C for 6 minutes. After heating, tubes were placed on ice for at least 1 minute prior to adding to RT-LAMP reactions.

The RT-LAMP and RT-qPCR assay results were compared for swab and saliva samples. To run samples in RT-qPCR, RNA was extracted from 200 μL of heated swab and saliva samples using the PureLink Viral RNA/DNA extraction kit (Invitrogen 12280050) per manufacturer's instructions, including the proteinase K lysis step and with a final elution into 50 μL of nuclease-free water. The RT-qPCR reactions were assembled using the Centers for Disease Control and Prevention SARS-CoV-2 assay, including N1, N2, and RPP primers ordered from Integrated DNA Technologies, and the TaqPath RNA-to-Ct RT-qPCR kit (Applied Biosystems 4392653) per manufacturer's instructions.

RESULTS AND DISCUSSION

After initial experiments validating the performance of the RT-LAMP reaction on the BioRad CFX96, attempts to perform the same RT-LAMP reactions on the Axxin T8-ISO resulted in false-positive amplification as early as 27 minutes (**Fig. 1**). False-positive formation occurs inconsistently on the T8-ISO, indicating spurious nonspecific product generation. False-positive results were obtained when identical reaction mixes were prepared in the clean reaction room, separated into 2 sealed 8-tube strips, and run concurrently on the 2 instruments, therefore ruling out the possibility of environmental contamination in only the T8-ISO strips. The presence of a heated lid on the BioRad CFX96 is a major difference from the T8-ISO, and we hypothesized that temperature heterogeneity—as well as local concentration increases at the reaction surface to due to evaporation—in reaction tubes incubated in an instrument without a heated lid may lead to nonspecific amplification.

To investigate the theory that false-positive amplification occurs in the absence of a heated lid, we performed RT-LAMP on the BioRad CFX96 with the heated lid set to 105°C and with the heated lid switched off. With the heated lid switched off, amplification was observed in no-template control samples, supporting the hypothesis that temperature heterogeneity within the reaction tube leads to spurious amplification (**Fig. 2A, B**). Furthermore, melt curve derivative peaks in the no-template control samples that are distinct from those of true products suggest that the amplification products generated in the absence of lid heating were due to non-template amplification (**Fig. 2D**). LAMP-based assays have previously been demonstrated without false-positive amplification in
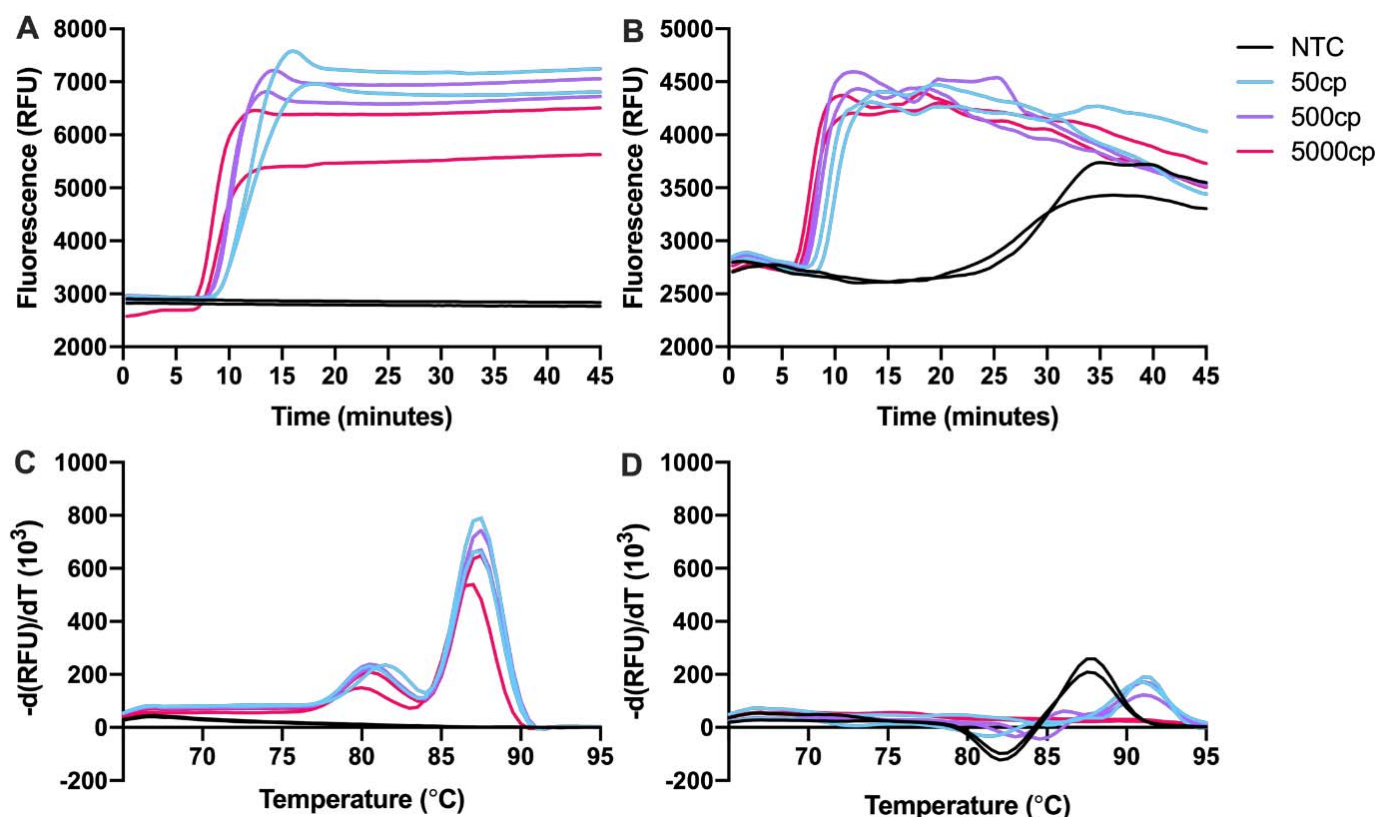
FIGURE 2

Fluorescence *vs*. time (A,B) and melt curve derivative data (C,D) for identical RT-LAMP reactions run on the BioRad CFX96 (A,C) with the heated lid set to 105°C and (B,D) without the heated lid. Reactions contain water (NTC), 50, 500, or 5000 copies of synthetic SARS-CoV-2 RNA.

instruments without heated lids, potentially indicating that the phenomenon observed in this report is due to a specific combination of primers, tubes, and instrumentation.

Four strategies were implemented to mitigate non-template amplification observed in the initial reactions on the T8-ISO: (1) overlaying the reaction with mineral oil, (2) including the enzyme *Tte* UvrD helicase in the reaction, (3) including mixing during the amplification reaction, and (4) performing a brief vortex and centrifuge prior to the reaction

T A B L E   1

Summary of strategies studied to eliminate false positives in no-template control samples and the resulting number of no-template control samples, out of 7, that returned a positive result

Mineral oil volume (μL)	Helicase	Mixing ball	Vortex + centrifuge?	No. of positives/ 7 NTCs
0	No	No	Yes	3
25	No	No	Yes	3
50	No	No	Yes	2
0	No	Yes	Yes	7
0	Yes	No	Yes	1
50	Yes	No	Yes	0
50	Yes	No	No	0
25	Yes	No	No	0

Abbreviation: NTC, no-template control.

rather than relying on mixing by pipetting. These strategies were evaluated alone, and in combination, by running 7 no-template control reactions (using nuclease-free water as the sample) and 1 positive control reaction (50 copies of control RNA) in a strip of 8 tubes. Representative results are shown in **Fig. 3** and results are summarized in **Table 1**.

The conditions that most reliably suppressed non-template amplification were *Tte* UvrD helicase included at 0.2 μL per 50-μL reaction together with 25 or 50 μL of mineral oil atop the reaction. These results were repeated 3 times in separate experiments for confirmation (results not shown).

Finally, clinical samples of varying sample type were evaluated on the Axxin T8 and the BioRad CFX96 with the optimized conditions for each instrument, respectively. Results were found to be highly consistent across the 2 instruments, with clinical samples that were also positive by PCR generally amplifying on both instruments within approximately 20 minutes, and clinical samples that were negative by PCR and no-template controls remaining negative (representative results and controls shown in **Fig. 4**).

Whereas false-positive events generally occurred close to or after 30 minutes of incubation, and true positives were amplified earlier than 30 minutes, as in the representative curves shown in **Fig. 3**, we were unable to define a precise time threshold that could reliably distinguish between true positives
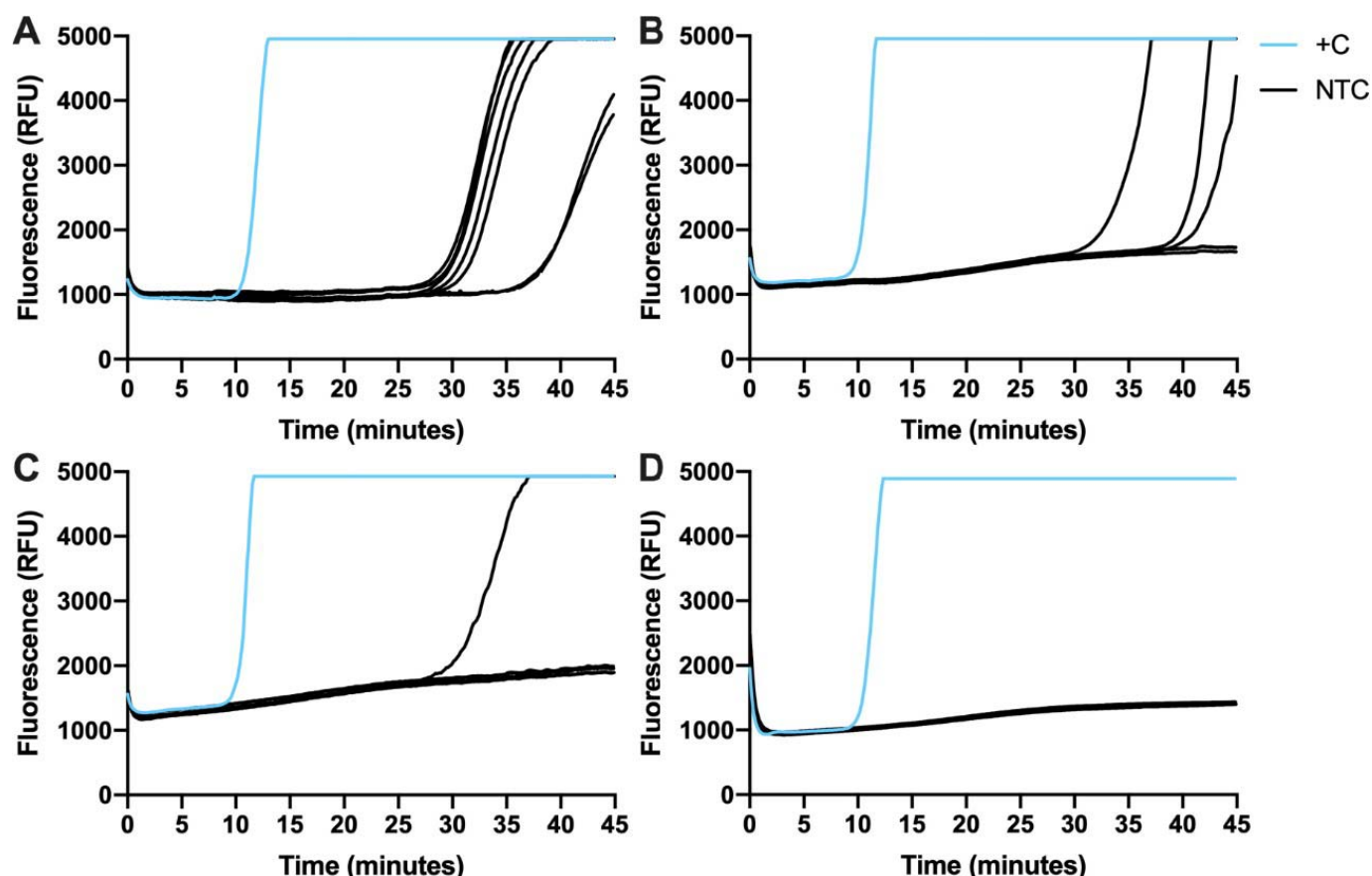
FIGURE 3

Fluorescence *vs.* time for 3 of the strategies studied to eliminate false positives in no-template control samples. RT-LAMP reactions were run on the Axxin T8-ISO and contain either nuclease-free water (NTC) or 50 copies of synthetic SARS-CoV-2 RNA (+C). (A) mixing ball; (B) 25 μL mineral oil overlayed; (C) 0.2 μL helicase per reaction; (D) 25 μL mineral oil + 0.2 μL helicase per reaction.
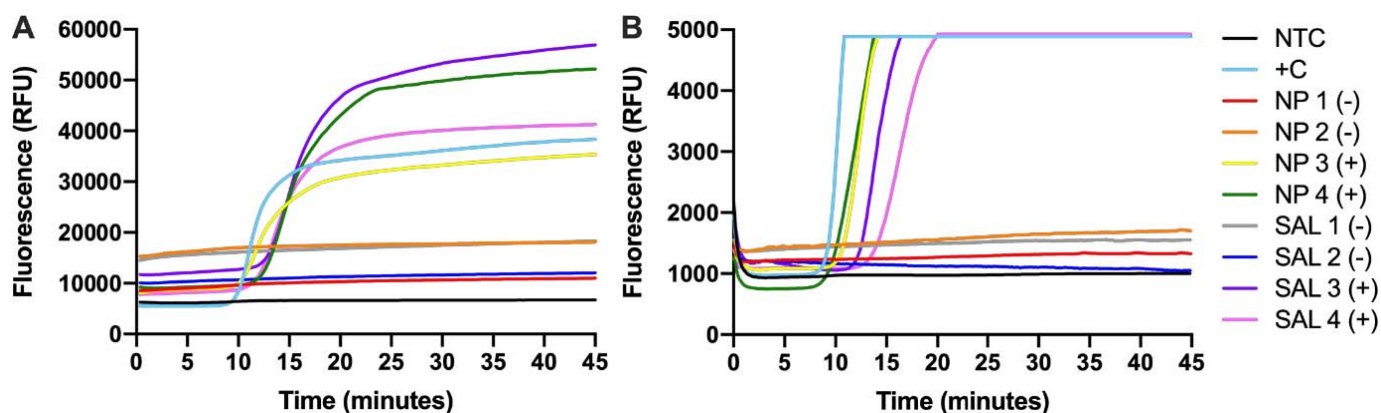

FIGURE 4

Fluorescence *vs.* time using 8 patient nasopharyngeal or saliva samples following the point-of-care lysis procedure and using (A) the manufacturer-recommended RT-LAMP protocol on the BioRad CFX96 and (B) the RT- LAMP protocol optimized for the Axxin T8-ISO). NP, nasopharyngeal samples; SAL, saliva samples; NTC, no-template control (water); +C, 500 total copies synthetic SARS-CoV-2 RNA. (+) and (−) refers to result obtained by RT-qPCR on the same samples.

and false positives without any of the interventions described. Particularly with low viral loads, sample amplification of RT-qPCR-confirmed positive samples can be delayed to the 30- to 45-minute range by inhibitors in the clinical matrix that remain in the reaction without an extraction or purification step (results not shown). In addition, when reactions are performed on a thermocycler, melt curve derivatives can provide additional insight on product specificity for late-amplifying samples, but melt curves cannot be acquired from the Axxin T8-ISO or other field-deployable molecular diagnostic instruments. Therefore, the approaches described here allow for longer incubation times to improve sensitivity without compromising specificity when using low-resource instruments.

CONCLUSION

Successful implementation of RT-LAMP for SARS-CoV-2 in point-of-care settings will remain useful in the foreseeable future to contain the virus spread as vaccines in development are validated for various populations at varying speeds and rollout reaches different areas of the globe. Aside from the COVID-19 pandemic, strategies to adapt LAMP-based assays to point-of-care fluorimeters may prove useful for other disease targets. Here, we have described challenges encountered when adapting an RT-LAMP assay to the Axxin T8-ISO isothermal fluorimeter and strategies used to eliminate false-positive events. Further optimization using lyophilized LAMP reagents and alternate strategies to seal reactions, such as low-temperature wax dots, could increase ease of implementation of similar assays and instruments. Using the optimized conditions described in this work, the Axxin T8-ISO could provide a promising alternative to traditional thermocyclers for identification of SARS-CoV-2 infections in settings with limited infrastructure and low throughput needs.

REFERENCES

1. JASON. Managing the risk from COVID-19 during a return to on-site university research. Available at: https://fas.org/irp/agency/dod/jason/covid-19.pdf. Published August 25, 2020. Accessed April 20, 2021.
2. Government of Ireland Department of Health. Report of the COVID-19 rapid testing group. Available at: https://www.gov.ie/en/publication/f50f0-report-of-the-covid-19-rapid-testing-group/. Updated April 1, 2021. Accessed May 19, 2021.
3. Bhadra S, Riedel TE, Lakhotia S, Tran ND, Ellington AD. High-surety isothermal amplification and detection of SARS-CoV-2, including with crude enzymes. *bioRxiv*. 2020. https://doi.org/10.1101/2020.04.13.039941
4. Ido Y, Hashimoto M, Yatsushiro S, *et al*. Loop-mediated isothermal amplification in microchambers on a cell micro-array chip for identification of plasmodium species. *J Parasitol*. 2019:105:69–74. https://doi.org/10.1645/18-107
5. Wu H, Qian C, Wu C, *et al*. End-point dual specific detection of nucleic acids using CRISPR/Cas12a based portable biosensor. *Biosens Bioelectron*. 2020;157:112153. https://doi.org/10.1016/j.bios.2020.112153
6. An L, Tang W, Ranalli TA, Kim HJ, Wytiaz J, Kong H. Characterization of a thermostable UvrD helicase and its participation in helicase-dependent amplification. *J Biol Chem*. 2005;280:28952–28958. https://doi.org/10.1074/jbc.M503096200
7. Mauris J, Evans TC. A human PMS2 homologue from *Aquifex aeolicus* stimulates an ATP-dependent DNA helicase. *J Biol Chem*. 2010;285:11087–11092. https://doi.org/10.1074/jbc.M109.050955
8. Vincent M, Xu Y, Kong H. Helicase-dependent isothermal DNA amplification. *EMBO Reports*. 2004;5:795–800. https://doi.org/10.1038/sj.embor.7400200
9. Tanner N, Evans TC Jr, inventors; New England Biolabs, Inc, assignee. Helicase suppression of non-template amplification. US patent 9,920,358 B2. March 20, 2018.
10. Zaczek-Moczydłowska MA, Mohamed-Smith L, Toldrà A, *et al*. A single-tube HNB-based loop-mediated isothermal amplification for the robust detection of the Ostreid herpesvirus 1. *Int J Mol Sci*. 2020;21:1–15. https://doi.org/10.3390/ijms21186605
11. Lillis L, Siverson J, Lee A, *et al*. Factors influencing recombinase polymerase amplification (RPA) assay outcomes at point of care. *Mol Cell Probes*. 2016;30:74–78. https://doi.org/10.1016/j.mcp.2016.01.009
12. Natoli ME, Chang MM, Kundrod KA, *et al*. Allele-specific recombinase polymerase amplification to detect sickle cell disease in low-resource settings. *Anal Chem*. 2021;93:4840. https://doi.org/10.1021/acs.analchem.0c04191
13. Moody C, Newell H, Viljoen H. A mathematical model of recombinase polymerase amplification under continuously stirred conditions. *Biochem Eng J*. 2016;112:193–201. https://doi.org/10.1016/j.bej.2016.04.017
14. Zhang Y, Ren G, Buss J, Barry AJ, Patton GC, Tanner NA. Enhancing colorimetric loop-mediated isothermal amplification speed and sensitivity with guanidine chloride. *Biotechniques*. 2020;69(3):178–185. https://doi.org/10.2144/btn-2020-0078
15. Rabe BA, Cepko C. SARS-CoV-2 detection using isothermal amplification and a rapid, inexpensive protocol for sample inactivation and purification. *Proc Natl Acad Sci U S A*. 2020;117(39):24450–24458. https://doi.org/10.1073/pnas.2011221117
16. Tanner N. *Optimized integration of New England Biolabs ® Loop-Mediated Isothermal Amplification (LAMP) reagents with Axxin ISO instruments*. Available at: https://www.neb.com/-/media/nebus/files/application-notes/appnote_integration_of_neb_lamp_reagents_with_axxin_iso_instruments.pdf?rev=c57e794a276e437fbffe62c08317706e. Updated 2019. Accessed November 28, 2021.